The role of cell surface (co)-receptors in the recognition of *Phytophthora infestans* effectors in wild potato

MSc Minor Thesis

By: Yeshiwas Alemnew Abate

Laboratory of Plant Breeding

Wageningen University and Research, the Netherlands

August 2016

The role of cell surface (co)-receptors in the recognition of *Phytophthora infestans* effectors in wild potato

MSc Minor Thesis report (PBR-80424)

By: Yeshiwas Alemnew Abate

Reg. no. 840227003070

Supervisor: Emmanouil Domazakis

Examiner: dr.ir. Vivianne Vleeshouwers

Prof.dr.ir. Evert Jacobsen

Laboratory of Plant Breeding

Wageningen University and Research, the Netherlands

August 2016

Table of Contents

Abbreviations and acronyms....iii

Abstract....iv

Acknowledgments....v

Introduction....1

- Potato and its production....1
- Potato diseases....1
- Plant defence mechanisms and breeding for late blight resistance....3
- *Phytophthora* apoplastic effectors and plant surface receptors....5
- Research questions....7

Materials and methods....8

- Plant materials....8
- Protein expression and purification....8
- SDS-PAGE and immunoblotting....10
- PVX Agroinfiltration....11
- Quantification of silencing....11
- Infiltration of effector proteins and scoring....13

Results....14

- Expression of recombinant effector proteins in yeast....14
- PVX based VIGS is effective in wild *Solanum*....15
- Gene expression analysis revealed effectiveness of PVX based VIGS in wild potato genotypes....18
- Variable effector response was observed in wild *Solanum* genotypes....19
- Infiltration of effector proteins at 1µM or 2µM concentrations does not show difference in inducing cell-death response in wild potato genotypes....20
- Protein infiltration and PVX agroinfection showed variable cell death response....21

Discussion....23

- Phenotypic and molecular quantification of *PDS* silencing levels in wild potato genotypes....23
- Utilization of VIGS to investigate co-receptor function in wild potato genotypes....24
- SOBIR1 and SERK3 co-receptors are not required for recognition of the Scr74 effector protein variants in wild potato genotypes....25
- INF1 and EPI effector proteins does not induce cell death response in wild potato genotypes....25

References....27

Appendices....30

- Appendix A: Levels of HisHA protein expression and quantitative estimation....30
- Appendix B: Estimation of *PDS* gene expression level....31
- Appendix C: Cell death responses of protein infiltration and PVX agroinfection experiments....33

Abbreviations and acronyms

AVR	Avirulence
CC-NB-LRR	Coiled-Coil Nucleotide-Binding Leucine-Rich Repeat
ETI	Effector Triggered Immunity
ETS	Effector Triggered Susceptibility
HR	Hyper sensitive Response
PAMP	Pathogen Associated Molecular Patterns
P. infestans	*Phytophthora infestans*
PR	Pathogenesis Related
PTI	PAMP Triggered Immunity
PVX	Potato Virus X
R gene	Resistance Gene
RLK	Receptor Like-Proteins
RLP	Receptor Like-Kinase
RxLR	Arginine-any amino acid-Leucine-Arginine
VIGS	Virus Induced Gene Silencing

Abstract

Potato production is being increasingly threatened by late blight disease, caused by the oomycete Phytophthora infestans. The two major classes of pathogen effectors, the intracellular (cytoplasmic) and extracellular (apoplastic) are also found in oomycetes. Oomycetes produce a number of apoplastic effectors to overcome the action of host defence as well as acting as an avirulence factor in Solanum species. To defend themselves against such pathogen effectors, plants also deploy two layers of defence, the NB-LRR class of immune receptors at the cytoplasm and the PRR at the cell surface. Plant surface receptors function in association with surface co-receptors for downstream signalling to activate defence response at the cell surface. This research investigated the suitability of PVX based VIGS for transient loss of function to assess the involvement of SOBIR1 and SERK3 cell surface co-receptors in wild potato germplasm in the response to apoplastic effectors. We produced such apoplastic effectors from yeast strains expressing recombinant effector proteins of Scr74 (three variants), EPI1, EPIC2B and INF1. These effector proteins were infiltrated in PVX based VIGS treated genotypes. In this research we demonstrated that PVX based VIGS can be effectively employed in wild potato germplasm for transient loss of gene function. As the same time, we identified that SOBIR1 and SERK3 surface co-receptors are not required for the recognition of three Scr74 variants. Though we obtained preliminary results with this research, we recommend furthermore investigation and analysis with more biological replications.

Acknowledgments

First of all, I would like to thank my supervisor **Emmanouil Domazakis**, firstly for your kindness to allowing me to work with you. I owe special thanks for your detailed support and technical guidance to keep me on track of the science. Your in-depth explanations and supervisions and the constructive feedbacks on my proposal and report writing always inspired me to work hard.

I would like also to express my heartfelt acknowledgment to **Vivianne Vleeshouwers** for giving me the opportunity to work in your research group. I also thank you for your valuable feedbacks and suggestions in my whole research processes. I also would like to thank **Doret Wouters** for helping out me in RNA isolation and arranging greenhouse facilities for my plants. Besides, I would like to thank the greenhouse staff who took care of my plants. Last but not least, I would like to thank everyone in laboratory of Plant Breeding for the wonderful time I had during my thesis.

Introduction

Potato and its production

Potato was initially domesticated in South American Andes region, and nowadays its cultivation is distributed to extensive areas of the world (Harris 2012) encompassing more countries than any other crop next to maize (Horton and Sawyer 1985). The tuber bearing cultivated species of potato is *Solanum tuberosum* L. (Haverkort et al. 2009). Potato's worldwide importance as food crop ranks third next to rice and wheat and its consumption throughout the world accounts for more than a billion people indicating the significant role it plays in food security for the increasing world population. Similarly, global areas devoted to potato production reach more than 19 million hectares and 325 million tonnes of total world production indicating a productivity of 17 tonnes per hectare (Birch et al. 2012).

The overall production of potato in developing countries shows increasing trend which can be accounted to changes in consumption cultures as a result of increasing urbanization with evolution of fast serving restaurants and supermarkets for processed potato products. However, this increment is due to increase in the areas devoted to potato production but the yielding trend is declining. On the other hand, the production trend and per capita consumption is decreasing in developed countries specially in Europe (Haverkort et al. 2009). In addition to its vital importance as a food crop, potato has also reputable history in non-food applications. For instance, in terms of industrial raw material, potato's starch has long been used in adhesive and textile industries (Kraak 1992).

Potato diseases

Although potato becomes a worldwide important crop and vital for food security, its production is facing serious challenges due to an ongoing occurrence of diseases and pests. If the yield reduction due to pests and diseases could have been controlled by at least a quarter, the worldwide potential production of potato could exceed over 400 million tonnes per annum (Gebhardt and Valkonen 2001). Potato late blight caused by the oomycete *Phytophthora infestans* remained the most economically important disease threatening the worldwide potato production (Birch et al. 2012). If environmental conditions are conducive and the disease occurs in early season of potato's growth, late blight can cause up to 80%

yield reductions. Consequently, developed countries spend 10-20% of total production costs towards chemical fungicides for the control of late blight while developing countries harvest an estimate of about 25% of the yields of developed countries due to lack of access for such chemical fungicides (Haverkort et al. 2009). In terms of global level financial losses and economic costs, (Haverkort et al. 2009) estimated that about €5.2 million is spent annually for the control of late blight disease in potato.

Oomycetes were formerly categorized under filamentous fungi group. However, advances in phylogenetic and biochemical studies enabled to identify oomycetes as an independent group of plant pathogens different from fungi. Accordingly, oomycetes are categorized as a distinctive group of pathogens more related to brown algae (Heterokont algae) with characteristic mechanism of interaction and ability to infect plants (Kamoun, Huitema, and Vleeshouwers 1999). Evolutionary relationships of the different eukaryotic organisms is presented in the following phylogenetic tree (Fig. 1) indicating that oomycetes are independent clades of plant pathogens.

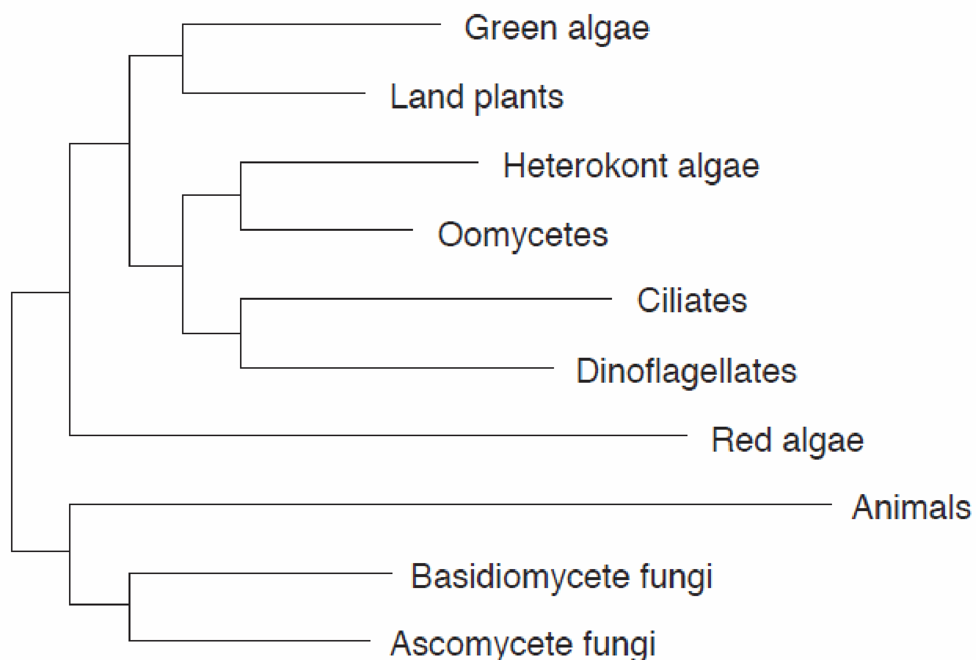


Figure 1: Phylogenetic tree showing oomycetes' evolutionary relationships with eukaryotes confirming that oomycetes have little share with the fungi groups, instead more related to the Heterokont algae. Adopted from (Kamoun, Huitema, and Vleeshouwers 1999).

Plant defence mechanisms and breeding for late blight resistance

Plants deploy two classes of immune receptors localizing on the cell surface and in the cell cytoplasm, to defend themselves against pathogens (Jones and Dangl 2006). The first line of defence recognizes conserved pathogen-associated molecular patterns (PAMPs) by cell surface receptors (Fig. 2) which are called pattern recognition receptors (PRR). The PAMPs are abundant pathogen molecular patterns conserved in, and crucial to the survival or pathogenicity of microbes. PAMPs, when exposed at plant cell surface, can be recognized as non-self molecules (Medzhitov and Janeway 1997). The second line of defence depends on the recognition of pathogen avirulence proteins (e.g. RxLR effectors for *Phytophthora*) by resistance (R) proteins. The R proteins belong to the coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) domain of plant immune receptors (Jones and Dangl 2006). These NB-LRR receptors recognize effectors that are delivered intracellularly, whereas cell surface receptors recognize pathogen effectors delivered into the apoplast (Fig. 2) targeting extracellular or membrane proteins (Stotz et al. 2014).

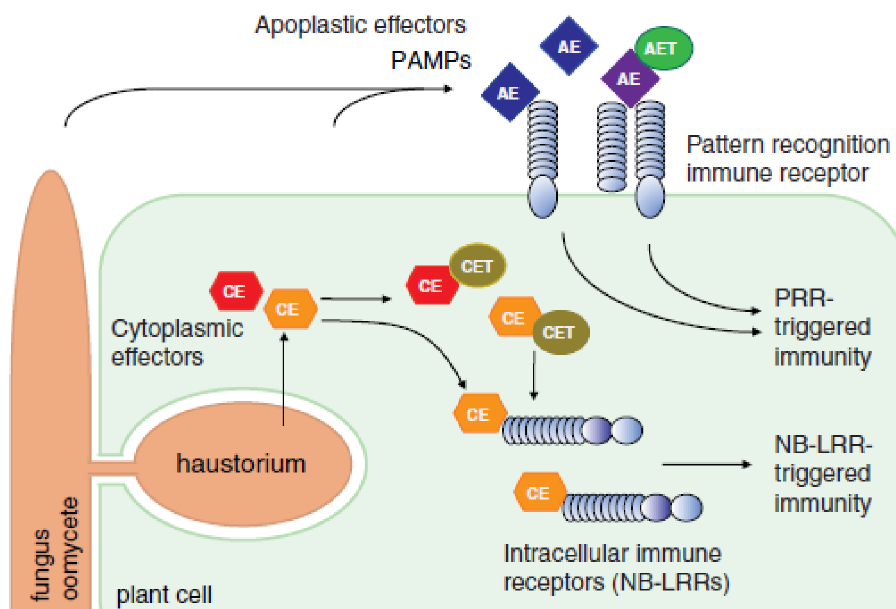


Figure 2: The two layers of plant defence and concept of effector-receptor interactions in plant immunity. Effectors delivered at the host interface are apoplastic effectors (AE) or inside the cell are cytoplasmic effectors (CE). Such effectors target and manipulate different host proteins and the targets are called apoplastic effector targets (AET) and cytoplasmic effector targets (CET) depending on localization in host cell. The PRRs detect PAMPs, the apoplastic effectors and apoplastic effector–target interaction initiate PRR triggered immunity (PTI). The NB-LRR induces NB-LRR-triggered immunity (ETI) on recognition of cytoplasmic effectors and/or cytoplasmic effectors–target interactions. Adopted from (Win et al. 2012).

Pathogens produce extracellular effectors to overcome initial defence related proteins, the PAMP triggered immunity (PTI), whereas intracellular effectors suppress signal transduction and regulation mechanisms to overcome effector triggered immunity (ETI) associated with plant defence attempts (Hein et al. 2009). PAMPs and other elicitors of oomycetes trigger PTI at the cell surfaces of hosts. Conversely, oomycetes deploy effectors to suppress such host defence, leading to effector triggered susceptibility (ETS). In turn, R proteins recognize intracellular effectors (AVR proteins) at cell cytoplasm (Fig. 3) and activate ETI, leading to hypersensitive response (HR) towards resistance against oomycetes. This follows a continuum in the oomycete-plant interaction zig-zag model (Fig. 3) with co-evolution of plant defence response and pathogen virulence strains (Hein et al. 2009).

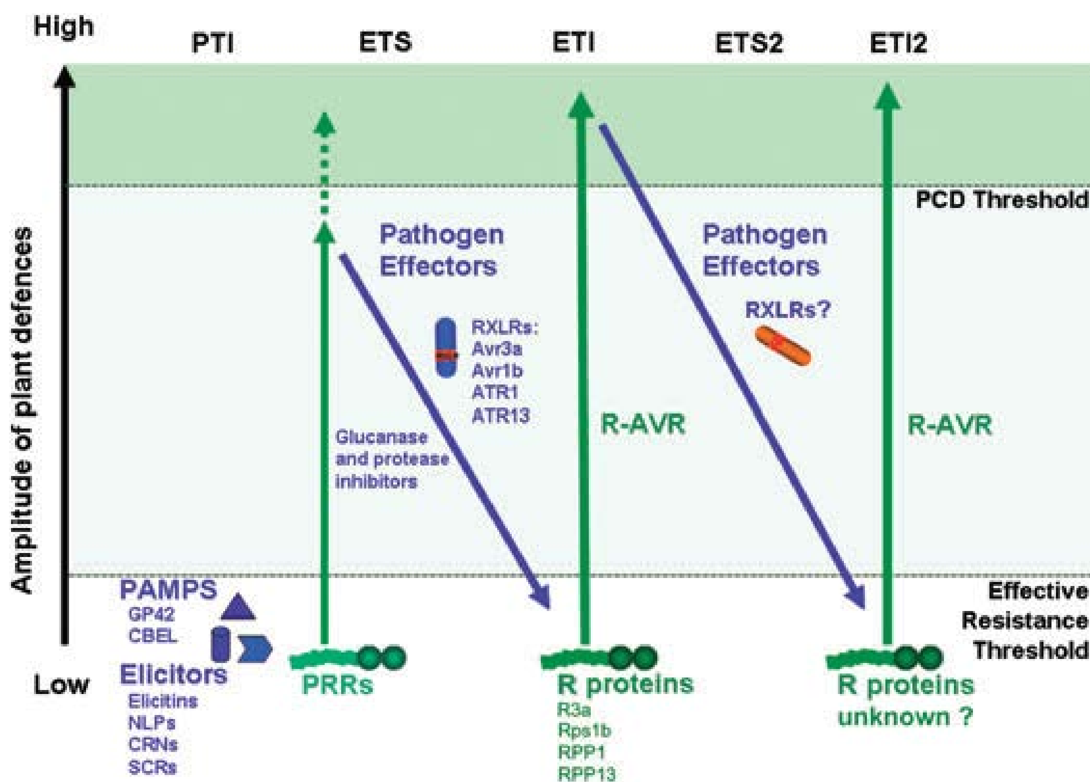


Figure 3: Schematic presentation of oomycete-plant interaction zig-zag model showing continuous and co-evolving interactions, resulting in a threshold of plant defence and resistance specificities. Adopted from (Hein et al. 2009).

Potato breeding for late blight resistance so far relied on *R* genes which belong to the NB-LRR class of immune receptors (Du et al. 2015). However, *P. infestans* genotypes undergo rapid evolution leading to host jumps and specialization to avoid recognition by plant

intracellular immune receptors (Raffaele et al. 2010). This *R* gene based resistance reportedly had been broken-down rapidly due to the evolution of corresponding *P. infestans* virulence races (Jansky 2000). Consequently, several studies described that pyramiding of multiple *P. infestans R* genes contributed to durable and broad spectrum resistance against late blight (Kim et al. 2012, Zhu et al. 2012, Haesaert et al. 2015). Similarly, Du et al. (2015) reported that surface receptor proteins such as ELR provided greater resistance against secreted *P. infestans* elicitin, INF1, and introgression of such cell surface receptors into commercial cultivars could enhance resistance towards the devastating late blight disease in potato. As a result, they suggested that pyramiding of extracellular receptors with intracellular receptors would maximize resistance durability. Accordingly, exploring for surface receptor resistance genes against apoplastic effectors is important to incorporate them into *R* gene based breeding programs.

Phytophthora apoplastic effectors and plant surface receptors

The two major classes of pathogen effectors, the intracellular (cytoplasmic) and extracellular (apoplastic) are also identified in oomycetes (Hein et al. 2009). Oomycetes including *Phytophthora* species protect themselves from hydrolytic activity of plant PR proteins by secreting protease inhibitors, thereby enhancing host infection processes. A number of such extracellular protease inhibitors were described to enabling pathogens to overcome the action of host protease as well as acting as an avirulence factor in *Solanum* species(Kamoun et al. 1997). Serine protease inhibitors, EPI1 and EPI10 (Tian et al. 2004) and cysteine-like protease inhibitors, EPIC1 and EPIC2B (Tian et al. 2007) were functionally characterized as kazal-like extracellular protease inhibitor families in *P. infestans*. Kamoun et al. (1997) also isolated and characterized a secreted elicitin encoding INF1 protein from *P. infestans.* INF1 is among the well-studied elicitins and conserved in *Phytophthora* species by which the expression of *inf1* elicitin gene during *P. infestans*–potato interactions induces HR-like defence responses (Breen 2012). Small cysteine-rich protein family (SCR74), encoding 74-amino acids was also characterized by Liu et al. (2005), with similarity to PcF (*Phytophthora cactorum* f.sp. fragariae (Nicastro et al. 2009, Orsomando et al. 2003)) protease family. The Scr74 protein family is described as highly polymorphic with 21 different amino acid sequences identified. Furthermore, Liu et al. (2005) elucidated that gene duplication and

recombination led to functionally divergent *scr74* family in *P. infestans*. Though the function of SCR74 is not yet elucidated, studies indicated that such effector proteins (mentioned above) are secreted and up-regulated in host apoplast during infection process (Tian et al. 2007, Liu et al. 2005, Kamoun 2006).

On the other hand, host plants respond against apoplastic effectors by recognizing them via PRRs (surface receptors/apoplastic receptors). Surface receptors are characterized either by receptor-like proteins (RLP) or receptor-like kinases (RLK). RLKs constitute the largest group of surface receptors containing LRR domain, transmembrane domain and the cytoplasmic kinase domain, while RLPs form the second group of surface receptors, structurally similar to RLK but lack only the cytoplasmic kinase domain (Wang et al. 2008). Receptor-like proteins, such as ELR were identified as mediating broad spectrum extracellular recognition of elicitin proteins from *Phytophthora* species (Vleeshouwers et al. 2014, Du et al. 2015).

RLPs activate defence response at the cell surface, yet they do not contain a signalling domain. However, RLPs function in association with surface co-receptors for downstream signalling. For example, reports showed that the receptor-like protein, ELR mediates broad spectrum resistance and functions in association with SERK3 (Du et al. 2015) and SOBIR1 (Emmanouil Domazakis, unpublished data). SERK3 and SOBIR1 are among the identified cell surface co-receptors and function to mediate downstream signal transduction for surface receptors. Chaparro-Garcia et al. (2011) also studied that SERK3 contributes to, and regulates immune response in *Nicotiana benthamiana* triggered against *P. infestans.* Furthermore, SOBIR1 is found in a complex with RLP and functions as a regulatory RLK for RLP-mediated immunity (Liebrand et al. 2013). Similarly, Liebrand, van den Burg, and Joosten (2014) studied that SOBIR1 is required specifically for receptor complexes containing RLP in tomato and *Arabidopsis thaliana,* while SERK3 is central regulator for both RLP and RLK in plasma membrane-associated receptor complexes. Moreover, these receptor like kinases (SERK3 and SOBIR1) are considered to be conserved throughout the plant kingdom (Liebrand, van den Burg, and Joosten 2014).

This study aimed into investigating the involvement of cell surface co-receptors in the response to apoplastic effectors in order to characterize extracellular resistance genes responsible for PRR-based plant defence. To do so, we employed virus induced gene silencing (VIGS) approach as a means for rapid assessment of the function of cell surface co-

receptor genes. VIGS is an increasing used technique to make transient loss of function of genes as a best alternative to stable transformation for assessing gene function (Baulcombe 1999). The significant application of VIGS is to study the function of a specific gene by silencing with reverse genetics approach at particular plant biological process (Senthil-Kumar and Mysore 2014). VIGS is also accredited for its applicability to employ reverse genetics approach that begins from known sequences of a gene to enabling identification of consequent phenotypic response *in planta* with relatively less labour and time (Sahu et al. 2012). Different viral vectors like potato virus X (PVX), tobacco rattle virus (TRV), and tomato golden mosaic virus (TGMV) etc. have been optimised for VIGS in different host species (Faivre-Rampant et al. 2004). Faivre-Rampant et al. (2004) demonstrated that PVX based VIGS is effectively utilized to activate VIGS response in both wild and cultivated *Solanum* species.

This research was carried out with PVX based VIGS for examining the function of cell surface receptor in the recognition of apoplastic effectors in wild potato germplasm. The research studied the silencing level and the subsequent loss of function of SOBIR1 and SERK3 cell surface co-receptors comparing with the silencing of controls (*PDS* and *GUS* genes). Accordingly, this research demonstrated that VIGS can be effectively utilized to triggering gene silencing in wild potato genotypes. In this thesis research, the outcome of the loss of function of these receptors in relation cell death response could not be completed due to the time limit of the thesis period. The study put ongoing research to exploring the roles of such surface receptors in response to apoplastic effectors.

Research questions

i. Can VIGS be utilized as a tool to study effector responses in wild potato germplasm?
ii. Does cell death response to apoplastic effectors require the co-receptors SERK3 and SOBIR1?

Materials and methods

Plant materials

Based on preliminary experiments, wild potato genotypes were selected for their response to specific *P. infestans* apoplastic effectors (based on PVX agroinfection data). A list of potato genotypes and the corresponding effector protein families are presented in table 1. Stock plants of selected genotypes were maintained *in vitro* by cutting plantlets and growing on MS20 medium (20 g/L sucrose, 4.4 g/L MS salts with vitamins, 8 g/L micro agar and pH 5.8, (Murashige and Skoog 1962)) under 24^{0}C temperature and 16h/8h light/dark climate room to induce roots and shoots as described by Du, Rietman, and Vleeshouwers (2014). Plants were propagated again on MS20 medium (as mentioned above) by cutting from the established stocks and grown *in vitro* for ±2 weeks. After rooting and shooting, plants were transferred to greenhouse at 16/8h light and dark photoperiods under standard soil conditions. These plants were grown in greenhouse for PVX agroinfection (VIGS treatment) and subsequent effector protein infiltration.

Table 1: Wild potato genotypes used in this study for PVX based VIGS with respective apoplastic effector recognition pattern (based on PVX data)

Solanum species	CBSG clone number	Effector family	Apoplastic effector members
Solanum microdontum	MCD360-1	INF1	INF1
S. verrucosum	VER922-1	Scr74	Scr74 G1/B3b
	VER989-4	Scr74	Scr74 A10/G1/B3b
	VER910-5	Scr74	Scr74G1
S. microdontum subsp gigantophyllum	GIG362-6	Scr74	Scr74B3b
S. hougassi	HOU654-1	Scr74	Scr74B3b
	HOU271-1	Scr74	Scr74G1/B3b
	HOU272-1	Scr74	Scr74G1
	HOU655-1	Scr74	Scr74B3b
S. huancabambense	HCB353-8	EPI	EPI1/EPIC2B

Protein expression and purification

The effector proteins studied in this thesis were INF1, Scr74 (Scr74-A10, Scr74-G1 and Scr74–B3b), EPI1 and EPIC2B (Table 1). *Pichia pastoris* yeast strains expressing these recombinant effector proteins (PichiaPink system) were available in the lab. Pre-cultures in adenine

auxotrophy selection media were inoculated in 25 mL of BMGY medium (10 g/L yeast, 20 g/L peptone, dissolved in MQ, 100 mL/L 1M potassium phosphate, 10 mL/L 10x yeast nitrogen base, 2mL/L 500x biotin, 100mL/L 10x glycerol, pH 6.0) in 250 mL baffled flask and incubated overnight at 28^0C shaking incubator (250–300 rpm). For the production of yeast biomass, 100–400 µL of the 25 mL cultures (based on visual cell density) were inoculated with 1 L BMGY in big flasks and incubated at 28^0C and 250-300 rpm shaking incubator until the OD_{600} reached 2-6. These cultures were centrifuged and pellets were suspended with 200 mL BMMY medium (10 g/L yeast, 20 g/L peptone, 100 mL/l 1M potassium phosphate, 10 mL/L 10x yeast nitrogen base, 2 mL/L 500x biotin, 100 mL/L 10x methanol, pH 6.0) for induction of protein expression. This suspension was incubated again at 28^0C on shaking incubator (300 rpm) for three days. Methanol was added every 24-hours to maintain induction and samples were taken before addition of methanol at each time point. After three days of expression, cells were harvested by centrifugation and the supernatant was stored at -80^0C until purification. For purification, proteins stored in -80^0C were defreezed and centrifuged. Then, the proteins were filter sterilized and concentrated with a pressurized concentrator (Amicon ultrafiltration device) technique. Concentration of proteins was performed by using filters with a size exclusion limit of 3kDa.

Concentrated proteins were purified with cation exchange (CEX) chromatography using SP Sepharose fast flow (GE Healthcare). The purification was performed by equilibrating the CEX medium (column beads) by passing 100 mL of acetic acid buffer (50 mM acetic acid, pH 4-5) (depending on the protein) through the column. The sterilized yeast supernatants were also diluted five-fold with the same buffer to decrease salt concentration and make the same pH as the column beads. Once, the beads were equilibrated, the protein sample was loaded into the column to bind target proteins into the column beads as a result of ion exchange, while unbound proteins and other impurities pass through with the buffer. After all sample volume was loaded and flow through is completed, the column was washed by passing 50 mL of acetic acid buffer. Finally, the bound proteins were eluted by passing 50 mL of elution buffer (10 mM Tris + 500 mM NaCl, pH 8) and individual 2 mL fractions of the proteins were collected in 2ml Eppendorf tubes. The relative protein concentrations of individual CEX elution fractions were measured with Nano-drop and fractions having ≥ 0.3mg/ml concentration were analysed by SDS-PAGE and western blotting.

SDS-PAGE and immunoblotting

To visualize expressed or purified proteins, SDS-PAGE and western blotting were carried out by preparing samples as 75 µl of protein sample with 25 µl of sample denaturation buffer (4x Laemmli) and boiled at 95^0C for 10 minutes (resulting in 1x Laemmli containing solution). Then 20 µl of the sample was loaded to TGX 4-20% gradient protein gel (BioRad) and ran for ±25 minutes at 250 V. Following SDS-PAGE, the gel was stained with Coomassie Brilliant Blue (CBB) or was used for western blot. For CBB staining, CBB staining solution (0.1% CBB R-250, 40% MeOH, 10% Glacial acetic acid) was added to the gel and the gel was incubated in a slow shaking agitator for 1 hour, then destained with destaining solution (10% MeOH, 7% Glacial acetic acid) overnight. Protein bands were analysed visually.

For western blotting, a transfer run was performed after SDS-PAGE gel run to transfer proteins from the gel into a PVDF (Polyvinylidene Difluoride) membrane. To do this, the membrane was cut to the dimension of the protein gel. The membrane was soaked in MeOH for activation and then the filter papers, the filter pads and the gel cassette case all were placed in a tray containing a transfer buffer (30 g/L TRIS, 140 g/L glycine, 10X diluted and 100 mL EtOH added stored at -4^0C). Then, the gel sandwich was prepared by putting in the order of gel cassette (gray side down), filter pad, filter paper, the gel, and the membrane. The sandwich was completed by placing filter paper on the membrane, then the filter pad and packed with the cassette. Then the blotting was run at 100 V for 45 minutes to 1-hour. Once the transfer run was completed, the membrane was blocked with TBS-T solution (12.1 g/L TRIS pH 7.5, 146 g/L NaCl, then diluted 10x and 0.5 mL Tween-20 added for final 0.05% Tween-20 concentration) containing 5% skimmed milk for 30 minutes. The membrane was then transferred to a falcon tube containing 5 mL TBS-T with 1 µl anti-HA-HRP antibody (Miltenyi Biotech) facing the side of the proteins towards inside of the tube. This was incubated for 2 hours or overnight (at 4 ˚C) on a rotating cylinder. Subsequently, the membrane washed with TBS-T 3 times for 5 minutes in a petri dish. Finally, the expression of protein bands was observed by imaging the transfer membrane in G-box machine using chemiluminescent substrates (Pierce, SuperSignal West Femto, Thermo Scientific). Purified fractions showing protein expression in SDS-PAGE and western blotting were combined and stored at -80 ˚C for later use.

PVX Agroinfiltration

PVX vectors were constructed targeting the silencing of *SERK3* and *SOBIR1* surface receptors and the phytoene desaturase (*PDS*) gene. The PVX-VIGS constructs used in this study include PVX-*SERK3*, PVX-*SOBIR1* as treatments and PVX-*GUS* and PVX-*PDS* as negative and positive controls respectively. Gene fragments (around 240 bp long) amplified from the open reading frame of the respective genes have been cloned into the gateway-compatible PVX vector pGWC-PVX (Valli, Dujovny, and García 2008).

The constructs containing the genes to be silenced were individually mobilized with *A. tumefaciens.* For agroinfiltration, *Agrobacterium* cultures were grown in LB medium with antibiotics. *Agrobacterium* cells from glycerol stock were inoculated in LB medium with antibiotics (kanamycin 50 µg/mL and chloramphenicol 34 µg/mL) in 10ml tube and grown overnight at 200rpm and 28^{0}C incubator (day 1). Estimated amount of these cultures depending on the growth were transferred to 50ml falcon tubes and inoculated in 15 mL YEB (5 g/L beaf extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 2mL/L 1M $MgSO_4$) with 1.5 µl acetosyringone including antibiotics (kanamycin 50 µg/mL), and 150 µl 1M MES and incubated overnight at 200 rpm and 28^{0}C incubator (day 2). Overnight grown cultures were centrifuged at 4000rpm for 10 minutes and pellets were suspended with fresh MMA (20 g/L sucrose, 5 g/L MS salt without vitamins, 1.95 g/L MES, pH 5.6 and 1mL/L 200mM acetosyringone). The final OD of this suspension was adjusted to 0.3 and then infiltrated into ±10 days old greenhouse grown plants (day 3). Agroinfiltration was performed in two leaves per plant using a needleless syringe on the underside of leaves covering the whole leaf area plus a small amount of inoculum was drenched at the crown region. The experiment was carried out in five plants per VIGS treatment per genotype for the three constructs (PVX-*GUS,* PVX-*SOBIR1* and PVX-*SERK3)* and two plants per genotype for PVX-*PDS* construct.

Quantification of silencing

The silencing situation of the gene of interest was monitored by following up the phenotype of PVX-*PDS* treated plants and by analysing the expression level of PDS gene by RT-qPCR. Leaf samples were also collected from third and fourth leaves from the top at ±21 days of VIGS treatment for RNA isolation and subsequent RT-qPCR analysis.

The collected leaves were frozen in liquid nitrogen and placed at -80 ^{0}C. Frozen leaf samples were ground in a tissue Lyser II (Qiagen) and RNA was isolated with RNeasy plant mini kit (Qiagen). The RNA was treated with DNase I according to the manufacturer instructions (Invitrogen) to eliminate any genomic DNA in the sample. Then cDNA was synthesised using iScript cDNA synthesis kit (BioRad).

RT-qPCR was performed in a total reaction volume of 10µl (5µl SYBR green buffer, 1µl forward primer, 1µl reverse primer, 2µl MQ water, and 1µl cDNA). The qPCR program ran at 95^{0}C initial denaturation for 3minutes followed by 40 cycles of 15 seconds denaturation at 95^{0}C, 1minute of annealing at 60^{0}C and 10seconds of extension at 95^{0}C. Approximately, 148 bp amplicon size of potato elongation factor (StEF1α, the housekeeping gene) was used to compare the level of expression of the gene of interest to be silenced. List of the primers and specificity conditions is presented in table 2. The level of silencing of the *PDS* gene was quantified by comparing the level of expression of the PDS gene in PVX-*PDS* silenced and PVX-*GUS* treated plants.

Table 2: List of primers used in semi-quantitative qPCR and RT-qPCR with their specificity conditions.

Primer name	Sequence (5'->3')	Product length (bp)	Annealing temperature(^{0}c)
VIGS-StPDS qPCR Fwd + Rev	GACAATACAGTTAACTATTTGGAGGC	335	60
	GCAAATATCATTGAATGTTCCTTC		
VIGS-StSOBIR1 qPCR Fwd + Rev	GAATTAGATTGGTTGGGACGA	322	60
	CCTTTCCGATAACCAACACAG		
VIGS-StSOBIR1-like qPCR Fwd + Rev	GCACGTCACCGAATTGC	315	60
	AGATGGAAGCTTTCCCATAAC		
VIGS-StSERK3A qPCR Fwd + Rev	CATTGTGATCCTAAAATTATTCATCG	338	60
	TCATATTTCTTGTCCTTCAGGAGTC		
VIGS-StSERK3B qPCR Fwd + Rev	TTGCGACCCTAAGATCATCC	337	60
	TTCATACTTCTCGTCCTTAAGAAGTC		
Stub/Nb EF1a qPCR Fwd + Rev	TGACCAAGATTGACAGGCGT	148	60
	GCAAAACGACCCAATGGTGG		

Infiltration of effector proteins and scoring

At 21 days of agroinfiltration (VIGS treatment), purified effector proteins were infiltrated on fully expanded third and fourth leaves from top (two leaves per plant) by using needless syringes. Each respective genotypes were infiltrated with members of corresponding protein families to differentiate responsive and non-responsive genotypes for specific effectors.

The level of response was quantified based on scale of 0%, 25%, 50%, 75%, and 100% of cell death from the infiltrated area. Per genotype, the average score of all infiltration spots was taken to quantify the overall response.

Results

Expression of recombinant effector proteins in yeast

Recombinant effector protein-producing *Pichia pastoris* clones (producing HisHA-tagged INF1, Scr74–A10, Scr74-G1, Scr74-B3b, EPI1 and EPIC2B) were available previously. Yeast cultures producing each protein were consecutively induced to produce each effector. The outcome of expression of these effector proteins was assessed by running SDA-PAGE and western blot analysis. To check expression level of proteins at different time points, proteins samples were taken at 24-hours intervals and analysed with SDS-PAGE and western blotting. SDS-PAGE and western blot analysis of samples taken at different time points (24, 48 and 72-hours) indicated sufficient expression at 48 and 72-hours. Proteins at 72-hour expression time were taken for this experiment (Supplementary Fig. 1). To obtain sufficient amounts of effector proteins, a scale up expression experiment was carried out by using flask cultures. Accordingly, we confirmed that each protein was produced successfully at reasonable levels based on SDS-PAGE and western blot (data not shown).

The proteins were purified with cation exchange (CEX) chromatography. The amount of purified proteins (concentrations) was quantified with bicinchoninic acid assay (BCA). Based on the BCA measurement, the highest amount of protein was obtained in Scr74B3b protein (Fig. 4). Furthermore, relatively sufficient amount of proteins was obtained for each effector protein in line with absolute standards (supplementary Fig. 2)

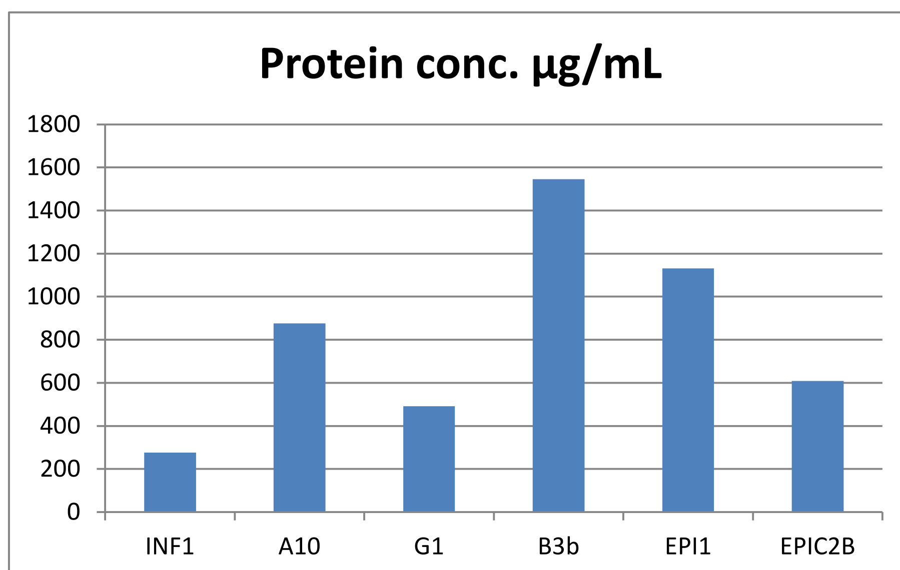
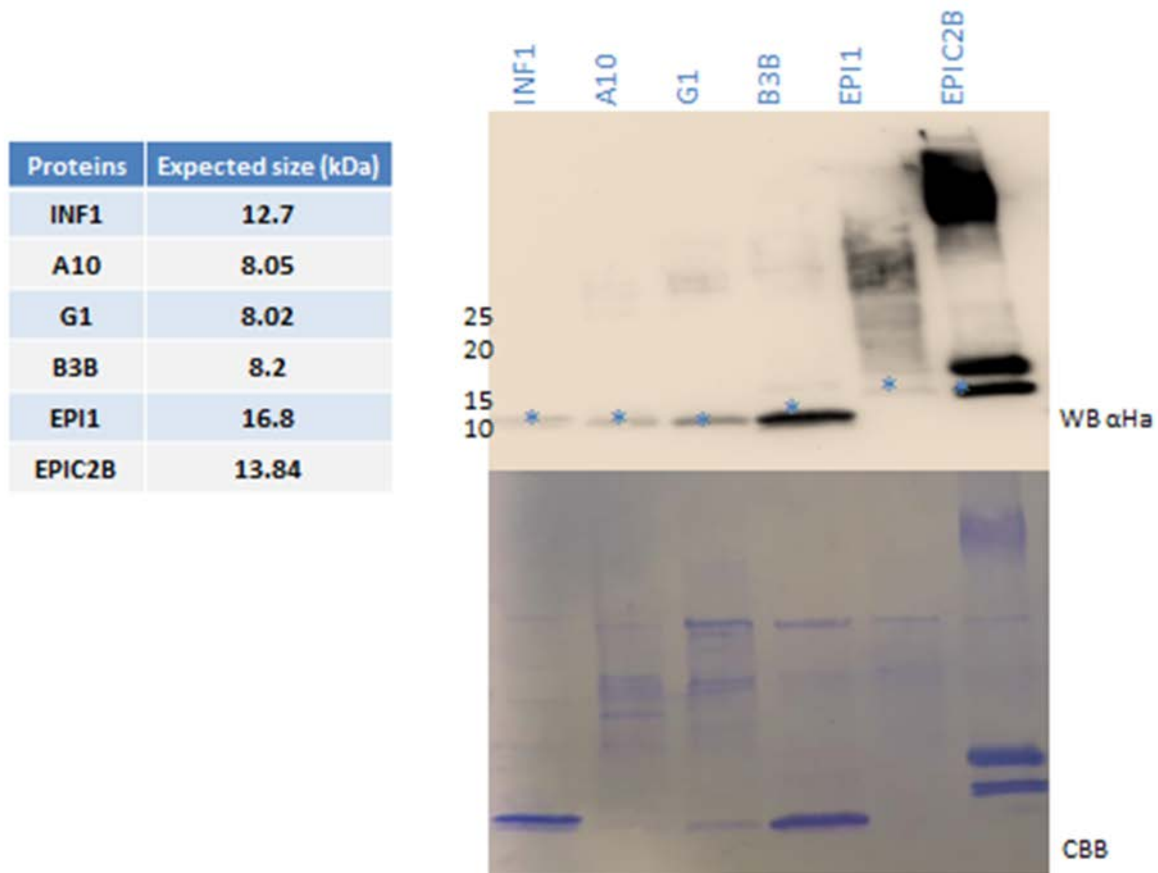


Figure: 4 Quantification of cation exchange (CEX) purified recombinant effectors using BCA.

The relative purity of purified proteins for each protein family was also analysed by SDS-PAGE and western blotting analysis. The highest relative purity (95%) was obtained in INF1 proteins while the lowest was observed in EPI1 proteins (Fig. 5).



Proteins	Expected size (kDa)
INF1	12.7
A10	8.05
G1	8.02
B3B	8.2
EPI1	16.8
EPIC2B	13.84

Figure 5: Purified proteins analysed with Western blotting and the membrane stained with CBB after imaging (upper image: Western blot, lower image: stained membrane with CBB) with expected sizes.

PVX based VIGS is effective in wild *Solanum*

To characterize the response of each genotypes to specific effector proteins, PVX-based virus induced gene silencing (VIGS) was employed because of theoretically better compatibility in wild potato genotypes than TRV-based VIGS. The progress of silencing was monitored by following up the photo bleaching symptom in PVX-*PDS* infected plants. The phenotypic symptom of *PDS* silenced plants was used as a visual marker for the silencing process. Because of high visible bleaching response to *PDS* silencing, *N. benthamiana* plants were also

treated with PVX-*PDS* and PVX-*GUS* for comparison of the silencing response with potato genotypes. In our experiment, phenotypic observation of plants showed that the PVX based VIGS induced active silencing process in wild potato genotypes (Fig 6, also supplementary Fig. 4). In some potato genotypes, the bleaching symptom was barely observable in the leaf veins. However, it could be noted that the high bleaching symptoms in *N. benthamiana* plants infected with PVX-*PDS* (control for symptom comparison) showed that our constructs were working well.

In this thesis research, genotypes VER922-1, HOU654-1 and HCB353-8 showed the least visible photo-bleaching in PVX-*PDS* infected plants than other genotypes. On the other hand, MCD360-1, GIG362-6, HOU271-1 and HOU272-1 showed the more clearly visible photo-bleaching symptoms (Fig 6).

Figure 6: Photo-bleaching symptoms of PVX-*PDS* treated plants compared to PVX-*GUS* treated counterparts suggesting the silencing level of *PDS* gene (pre-screening). Pictures were taken at 29 days post VIGS.

Gene expression analysis revealed effectiveness of PVX based VIGS in wild potato genotypes

The effectiveness of VIGS on potato genotypes was analysed in two approaches. Firstly, the level of silencing was estimated from the bleaching of leaves challenged by PVX-*PDS* compared to leaves challenged by PVX-*GUS*. Secondly, the level of silencing was quantified by comparing the level of expression of *PDS* gene in PVX-*PDS* and PVX-*GUS* treated plants with semi-quantitative PCR and RT-qPCR analysis. Semi-quantitative PCR (25 cycles) was performed as a preliminary estimate of the silencing levels. Potato elongation factor (StEF1α) was used as internal control to compare the expression levels of other genes (housekeeping gene) in both PVX-*PDS* and PVX-*GUS* treated plants.

In the case of semi-quantitative RT-PCR, seven out of ten genotypes showed weaker band strength in PVX-*PDS* treated plants than PVX-*GUS* treated counterparts (supplementary Fig. 3), which is suggestive of lower expression of the *PDS* gene. Similarly, RT-qPCR analysis indicated lower expression of *PDS* gene in seven out of ten genotypes of PVX-*PDS* treated plants compared to PVX-*GUS* treated ones (Fig. 8). In RT-qPCR analysis HCB353-8 genotype showed higher expression of the *PDS* gene in PVX-*PDS* treated plants indicating no silencing of the gene of interest. The highest level of silencing has been achieved 98% in VER989-4 genotype and the lowest 28% in HOU655-1 genotype. Overall, silencing level of the PDS gene in most of the genotypes ranged from 50 to 85%.

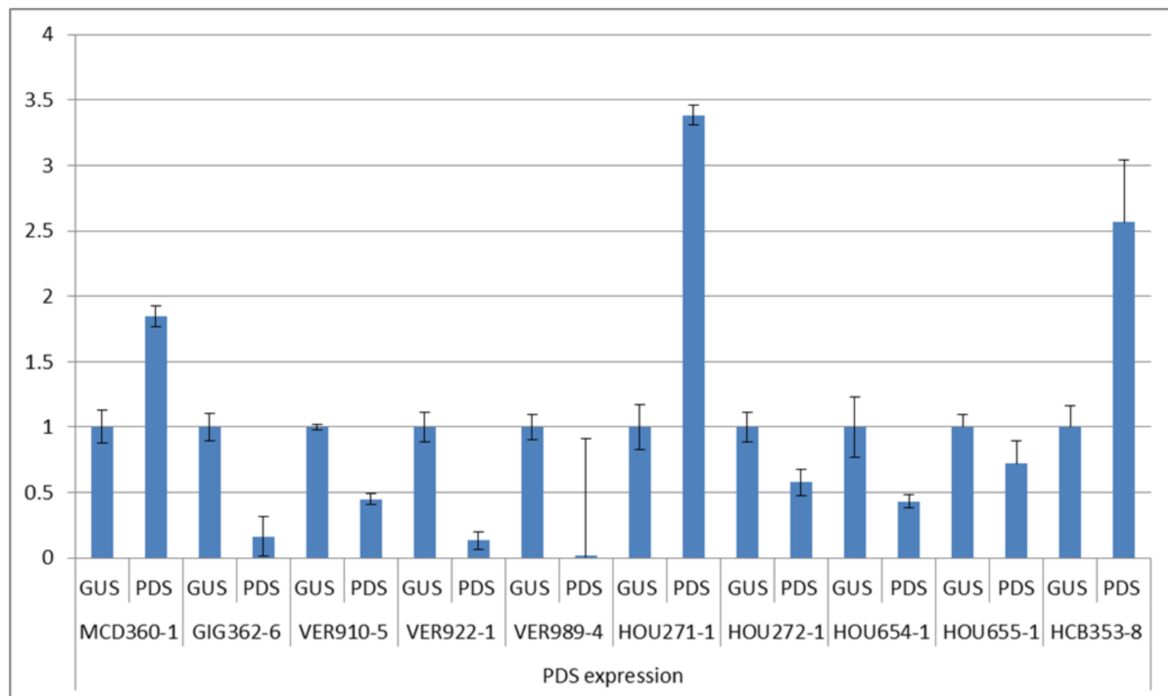


Figure 8: Relative quantification of the expression level of *PDS* gene in PVX-*PDS* and PVX-*GUS* treated plants by qRT-PCR. Error bars show standard deviation.

Variable effector response was observed in wild *Solanum* genotypes

Initially, pre-screening was carried out by treating plants only with PVX-*PDS* and PVX-*GUS* constructs using two plants per genotype for each construct. PVX-*GUS* infected plants were first tested to characterize responsive and non-responsive genotypes to specific effectors. Consequently, PVX-*GUS* infected plants were infiltrated with purified effector proteins. Although phenotypic bleaching was observed in some of the genotypes (suggestive of successful silencing), effector response of the genotypes could not consistently confirm the specificity of genotypes as stated in previous preliminary experiments.

Cell death response was scored at 8 dpi in percentage of 0 to 100% of the infiltrated areas, where 0 is no cell death response and 100% is full response (Fig. 7). The average score of each infiltration spots was taken as final value to characterize responsive and non-responsive genotypes to the specific effectors. Based on the pre-screening result, consistent response of genotypes against specific effector (s) could not be clearly identified. Therefore, for the main experiment, all Scr74 protein families are infiltrated to candidate genotypes (table 1).

Figure 7: Representative pictures of the cell death percentage scale used for scoring genotype responses against effector proteins (pictures of VER922-1 genotype infiltrated with 1 µM Scr74-B3b effector).

Infiltration of effector proteins at 1µM or 2µM concentrations does not show difference in inducing cell-death response in wild potato genotypes

To identify appropriate concentration of proteins, 1 µM and 2 µM concentrations were applied on the left and right side of leaves respectively (Fig. 9). Induction of cell death response against effector proteins was assessed in ±7 days of infiltration. The results from 1 µM and 2 µM protein infiltrations did not show significant difference in cell death response in all the studied genotypes (Fig. 9, table 3 and 4). Therefore, 1 µM concentration was chosen for further experiments.

Figure 9: Example leaves of PVX-*GUS* infected genotypes for responses against effector proteins infiltrated at 21 days post VIGS treatment (two leaves of each genotype). A) HCB353-8: EPI proteins, B) VER922-1: Scr74 proteins, (left side = 1 µM, right side = 2 µM). Pictures were taken at 8 dpi.

Table 3: Response of genotypes against specific effector members in 1 µM protein concentration

Genotypes/effectors	INF1	A10	G1	B3B	EPI1	EPIC2B
MCD360-1	9.4	x	x	x	x	x
VER922-1	x	100	56.3	43.8	x	x
VER989-4	x	18.8	37.5	12.5	x	x
VER910-5	x	43.8	25	0	x	x
GIG362-6	x	18.8	25	6.3	x	x
HOU654-1	x	12.5	12.5	0	x	x
HOU271-1	x	12.5	0	0	x	x
HOU272-1	x	0	0	0	x	x
HOU655-1	x	12.5	18.8	6.25	x	x
HCB353-8	x	x	x	x	62.5	31.3

Table 4: Response of genotypes against specific effector members in 2 µM protein concentration

Genotypes/effectors	INF1	Scr74-A10	Scr74-G1	Scr74-B3B	EPI1	EPIC2B
MCD360-1	25	x	x	x	x	x
VER922-1	x	62.5	37.5	6.3	x	x
VER989-4	x	18.8	6.3	6.3	x	x
VER910-5	x	81.3	43.8	18.8	x	x
GIG362-6	x	6.3	0	0	x	x
HOU654-1	x	6.3	12.5	12.5	x	x
HOU271-1	x	12.5	0	0	x	x
HOU272-1	x	0	0	0	x	x
HOU655-1	x	6.3	6.3	12.5	x	x
HCB353-8	x	x	x	x	100	31.3

Protein infiltration and PVX agroinfection showed variable cell death response

In our experiment, wild potato genotypes showed different pattern of cell death response to effector proteins compared to previous preliminary experiment results. Within this experiment, most of the genotypes studied didn't show cell death response to protein infiltration except *S. verrucosum* genotypes (data not shown). The *S. verrucosum* genotypes also induced less cell death response for protein infiltrations in PVX-*GUS* treated plants than in PVX-*SOBIR1* and PVX-*SERK3* treated counterparts (table 5). On the other hand, these genotypes showed high cell death response with PVX agroinfection (table 5 also supplementary Fig. 5) (PXV data from previous experiments).

Table 5: Cell death quantification results from protein infiltration compared to PVX agroinfection (data from previous studies) on selected responding genotypes. The PVX cell death scores were adjusted to 100 scale to match the protein infiltration cell death scoring.

Genotype/effectors	PVX-*GUS*				PVX-*SOBIR1*				PVX-*SERK3*				PVX agroinfection		
	Buffer	Scr74-A10	Scr74-G1	Scr74-B3b	Buffer	Scr74-A10	Scr74-G1	Scr74-B3b	Buffer	Scr74-A10	Scr74-G1	Scr74-B3b	Scr74-A10	Scr74-G1	Scr74-B3b
VER922-1	15.6	12.50	15.63	9.38	7.50	37.50	42.50	15.00	16.67	20.83	29.17	25.00	20	50	50
VER989-4	3.13	3.13	6.25	0.00	0.00	0.00	7.50	5.00	0.00	0.00	10.00	0.00	80	50	40
VER910-5	0	13.89	8.33	0.00	0.00	10.00	7.50	5.00	0.00	12.50	9.38	0.00	50	40	0

Discussion

Virus induced gene silencing (VIGS) is reported as rapid alternative to stable transformation to assess gene function assays (Baulcombe 1999). In this study, PVX based VIGS was investigated for the possibility of using in wild potato genotypes for transient loss of function for SOBIR1 and SERK3 cell surface co-receptors in the response to certain *P. infestans* apoplastic effectors. We examined the effectiveness of the PVX based VIGS on ten wild potato genotypes and analysed based on two approaches. Firstly, the level of silencing was estimated from the bleaching of leaves challenged by PVX-*PDS* compared to leaves challenged by PVX-*GUS*. Furthermore, the level of silencing was quantified by semi-quantitative qPCR and RT-PCR techniques. Furthermore, the response of VIGS treated genotypes to specific apoplastic effectors was investigated by infiltrating recombinant effector proteins such as Scr74 (three variants), EPI1, EPIC2B and INF1.

Phenotypic and molecular quantification of *PDS* silencing levels in wild potato genotypes

We used *PDS* silencing as an ideal silencing indicator because of the simplicity to visually phenotype *PDS* silencing in wild potato genotypes or in *N. benthamiana*. This estimation of *PDS* silencing offers helpful information to determining the efficiency of VIGS approach to use in silencing of a gene of interest for large scale screening (Faivre-Rampant et al. 2004). In our experiment, phenotypic observation of the silencing level in PVX-*PDS* challenged plants revealed varying level of photo-bleaching symptoms in the genotypes studied, indicating the different silencing levels of the *PDS* gene. We found that some of the genotypes exhibited barely visible photo bleaching responses along leaf veins and as patches of small spots on the foliar tissues. Faivre-Rampant et al. (2004) reported strong photo-bleaching with PVX based VIGS both in wild and cultivated potato genotypes challenged by PVX-*PDS*. However, we screened more number of genotypes which are different from the species studied by Faivre-Rampant et al. (2004) . On the other hand, other studies indicate that PVX based VIGS cannot infect meristem tissues and a proportion of leaf surface always remained green, thus causes difficulty of phenotyping the silencing situation (Ratcliff, Martin-Hernandez, and Baulcombe 2001).

In molecular gene expression analysis (RT-qPCR), most genotypes showed acceptable level of silencing ranging from 50-85% (Fig. 9). Only the genotypes MCD360-1, HOU271-1 and HCB353-8 showed higher expression of the *PDS* gene in PVX-*PDS* treated plants than the PVX-*GUS* counterparts (Fig. 9). The downregulation of *pds* expression in molecular analysis is reported to correlate with the visual photo-bleaching symptoms. In this research, genotype GIG362-6 showed more clear correlation of photo-bleaching with *pds* gene silencing. Although high silencing level of the *pds* gene was observed in RT-qPCR, the photo-bleaching symptom is not clearly visible in VER922-1, VER989-4 and HOU654-1 genotypes. On the other hand, MCD360-1 genotype showed clearly visible photo-bleaching but high mRNA transcript of PDS gene was also observed with RT-qPCR analysis. MCD360-1 genotype is more related with GIG362-6 genotype and at the same time, the phenotypic bleaching suggests that the *PDS* gene was silenced in this plant. Similarly, HOU271-1 genotype showed reasonable photo-bleaching but mRNA transcript of PDS gene was high. Therefore, this high level of *pds* expression in RT-qPCR analysis for MCD360-1 and HOU271-1 genotypes might be most likely due to sampling from a non-silenced leaf part.

Many studies reported that gene silencing is not uniform and requires large number of replicates (Senthil-Kumar and Mysore 2014). However, our result is from a single experiment without biological replications. Although, the main experiment was designed in three biological replications, the results of these experiments could not be analysed due to time limitations. Therefore, complete investigation and characterization of genotypes for PVX based VIGS and effector response would be achieved from follow-up experiment replications.

Utilization of VIGS to investigate co-receptor function in wild potato genotypes

In this experiment, we studied to characterizing wild potato genotypes for suitability in PVX based VIGS. Accordingly, our silencing results demonstrated that at least seven genotypes out of ten studied can be suitably used for PVX based VIGS. On the other hand, our protein infiltration results showed different pattern of cell death response than expected compared with cell death response index in PVX agroinfection. Genotypes which showed high cell

death index in PVX agroinfection induced less cell death response in protein infiltration and vice-versa. For example, VER922-1 genotype had low level of HR response in PVX agroinfection but higher cell death response in protein infiltration. This could be justified related to sensitivity of genotypes to the Scr74 effectors. Low HR index value in PVX Agroinfection could be related to high sensitivity of the genotype that the virus could not get time to spread more. Thus, we would say the less sensitivity could lead to high HR index in PVX agroinfection but results in low recognition for effector proteins.

SOBIR1 and SERK3 co-receptors are not required for recognition of the Scr74 effector protein variants in wild potato genotypes

We screened the response of wild potato genotypes to Scr74 protein family. The members of Scr74 protein family investigated include Scr74-A, Scr74-G1 and Scr74-B3b. The response of genotypes to protein infiltrations in PVX-*GUS*, PVX-*SOBIR1* and PVX-*SERK3* treated plants in this experiment indicated that SOBIR1 and SERK*3* co-receptors are not required for recognition of all the Scr74 effector protein variants studied. However, the response of the genotypes might be influenced by seasonal factors since it is performed in a long day summer. Thus, the experiment have to be repeated in a different season. This experiment is about starting information suggestive of the possibility to employ VIGS to investigating the function of cell surface co-receptor genes in wild potato genotypes. Nevertheless, more experiments must be performed to elucidate the response of genotypes to effector proteins upon silencing of the co-receptor genes.

INF1 and EPI effector proteins does not induce cell death response in wild potato genotypes

The Effector proteins INF1, EPI1 and EPIC2B were screened by infiltrating on PVX based VIGS treated genotypes. In our experiment, infiltration of these effector proteins didn't induced cell death response in all PVX-*GUS,* PVX-*SOBIR1* and PVX-*SERK3* treated corresponding genotypes. Though EPI1 and EPIC2B showed inconsistent cell death response in the different experiments, we couldn't observe any response to INF1 proteins. Accordingly, different factors might be involved for the non-responsiveness of the genotypes, one of which could

be environmental factor. Since this experiment is carried out during summer, the long day season might hinder the responsiveness of the genotypes. Therefore, to reveal the role of SOBIR1 and SERK3 co-receptors for the recognition of these effector proteins, the experiment need to be repeated in another time point.

References

Baulcombe, David C. 1999. "Fast forward genetics based on virus-induced gene silencing." *Current opinion in plant biology* 2 (2):109-113.

Birch, Paul RJ, Glenn Bryan, Brian Fenton, Eleanor M Gilroy, Ingo Hein, John T Jones, Ankush Prashar, Mark A Taylor, Lesley Torrance, and Ian K Toth. 2012. "Crops that feed the world 8: Potato: are the trends of increased global production sustainable?" *Food Security* 4 (4):477-508.

Breen, Susan Anne. 2012. "Investigation of the recognition and host target of the Phytophthora infestans effector PiAVR2." University of Dundee.

Chaparro-Garcia, Angela, Rachael C Wilkinson, Selena Gimenez-Ibanez, Kim Findlay, Michael D Coffey, Cyril Zipfel, John P Rathjen, Sophien Kamoun, and Sebastian Schornack. 2011. "The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen Phytophthora infestans in Nicotiana benthamiana." *PLoS One* 6 (1):e16608.

Du, Juan, Hendrik Rietman, and Vivianne GAA Vleeshouwers. 2014. "Agroinfiltration and PVX agroinfection in potato and Nicotiana benthamiana." *Journal of visualized experiments: JoVE* (83).

Du, Juan, Estelle Verzaux, Angela Chaparro-Garcia, Gerard Bijsterbosch, LC Paul Keizer, Ji Zhou, Thomas WH Liebrand, Conghua Xie, Francine Govers, and Silke Robatzek. 2015. "Elicitin recognition confers enhanced resistance to Phytophthora infestans in potato." *Nature Plants* 1 (4).

Faivre-Rampant, Odile, Eleanor M Gilroy, Katarina Hrubikova, Ingo Hein, Steve Millam, Gary J Loake, Paul Birch, Mark Taylor, and Christophe Lacomme. 2004. "Potato virus X-induced gene silencing in leaves and tubers of potato." *Plant Physiology* 134 (4):1308-1316.

Gebhardt, Christiane, and Jari PT Valkonen. 2001. "Organization of genes controlling disease resistance in the potato genome." *Annual review of phytopathology* 39 (1):79-102.

Haesaert, Geert, Jack H Vossen, René Custers, Marc De Loose, Anton Haverkort, Betty Heremans, Ronald Hutten, Geert Kessel, Sofie Landschoot, and Bart Van Droogenbroeck. 2015. "Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions." *Crop Protection* 77:163-175.

Harris, Paul M. 2012. *The potato crop: the scientific basis for improvement*: Springer Science & Business Media.

Haverkort, AJ, PC Struik, RGF Visser, and E Jacobsen. 2009. "Applied biotechnology to combat late blight in potato caused by Phytophthora infestans." *Potato Research* 52 (3):249-264.

Hein, Ingo, Eleanor M Gilroy, Miles R Armstrong, and Paul RJ Birch. 2009. "The zig-zag-zig in oomycete–plant interactions." *Molecular Plant Pathology* 10 (4):547-562.

Horton, Douglas, and Richard L Sawyer. 1985. "The potato as a world food crop, with special reference to developing areas." *Potato physiology*:1-34.

Jansky, Shelley. 2000. "Breeding for disease resistance in potato." *Plant Breeding Reviews* 19:69-156.

Jones, Jonathan DG, and Jeffery L Dangl. 2006. "The plant immune system." *Nature* 444 (7117):323-329.

Kamoun, Sophien. 2006. "A catalogue of the effector secretome of plant pathogenic oomycetes." *Phytopathology* 44 (1):41.

Kamoun, Sophien, Edgar Huitema, and Vivianne GAA Vleeshouwers. 1999. "Resistance to oomycetes: a general role for the hypersensitive response?" *Trends in Plant Science* 4 (5):196-200.

Kamoun, Sophien, Pieter van West, Anke J de Jong, Koen E de Groot, Vivianne GAA Vleeshouwers, and Francine Govers. 1997. "A gene encoding a protein elicitor of Phytophthora infestans is down-regulated during infection of potato." *Molecular Plant-Microbe Interactions* 10 (1):13-20.

Kim, Hyoun-Joung, Heung-Ryul Lee, Kwang-Ryong Jo, SM Mahdi Mortazavian, Dirk Jan Huigen, Bert Evenhuis, Geert Kessel, Richard GF Visser, Evert Jacobsen, and Jack H Vossen. 2012. "Broad spectrum late blight resistance in potato differential set plants MaR8 and MaR9 is conferred by multiple stacked R genes." *Theoretical and applied genetics* 124 (5):923-935.

Kraak, A. 1992. "Industrial applications of potato starch products." *Industrial Crops and Products* 1 (2):107-112.

Liebrand, Thomas WH, Grardy CM van den Berg, Zhao Zhang, Patrick Smit, Jan HG Cordewener, Antoine HP America, Jan Sklenar, Alexandra ME Jones, Wladimir IL Tameling, and Silke Robatzek. 2013. "Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection." *Proceedings of the National Academy of Sciences* 110 (24):10010-10015.

Liebrand, Thomas WH, Harrold A van den Burg, and Matthieu HAJ Joosten. 2014. "Two for all: receptor-associated kinases SOBIR1 and BAK1." *Trends in plant science* 19 (2):123-132.

Liu, Zhenyu, Jorunn IB Bos, Miles Armstrong, Stephen C Whisson, Luis da Cunha, Trudy Torto-Alalibo, Joe Win, Anna O Avrova, Frank Wright, and Paul RJ Birch. 2005. "Patterns of diversifying selection in the phytotoxin-like scr74 gene family of Phytophthora infestans." *Molecular biology and Evolution* 22 (3):659-672.

Medzhitov, Ruslan, and Charles A Janeway. 1997. "Innate immunity: impact on the adaptive immune response." *Current opinion in immunology* 9 (1):4-9.

Murashige, Toshio, and Folke Skoog. 1962. "A revised medium for rapid growth and bio assays with tobacco tissue cultures." *Physiologia plantarum* 15 (3):473-497.

Nicastro, Giuseppe, Giuseppe Orsomando, Elena Ferrari, Lucia Manconi, Filomena Desario, Adolfo Amici, Alessia Naso, Armando Carpaneto, Thelma A Pertinhez, and Silverio Ruggieri. 2009. "Solution structure of the phytotoxic protein PcF: the first characterized member of the Phytophthora PcF toxin family." *Protein Science* 18 (8):1786-1791.

Orsomando, G, M Lorenzi, E Ferrari, C De Chiara, A Spisni, and S Ruggieri. 2003. "PcF protein from Phytophthora cactorum and its recombinant homologue elicit phenylalanine ammonia lyase activation in tomato." *Cellular and Molecular Life Sciences CMLS* 60 (7):1470-1476.

Raffaele, Sylvain, Rhys A Farrer, Liliana M Cano, David J Studholme, Daniel MacLean, Marco Thines, Rays HY Jiang, Michael C Zody, Sridhara G Kunjeti, and Nicole M Donofrio. 2010. "Genome evolution following host jumps in the Irish potato famine pathogen lineage." *Science* 330 (6010):1540-1543.

Ratcliff, Frank, Ana Montserrat Martin-Hernandez, and David C Baulcombe. 2001. "Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing." *The Plant Journal* 25 (2):237-245.

Sahu, Pranav Pankaj, Swati Puranik, Moinuddin Khan, and Manoj Prasad. 2012. "Recent advances in tomato functional genomics: utilization of VIGS." *Protoplasma* 249 (4):1017-1027.

Senthil-Kumar, Muthappa, and Kirankumar S Mysore. 2014. "Tobacco rattle virus–based virus-induced gene silencing in Nicotiana benthamiana." *nature protocols* 9 (7):1549-1562.

Stotz, Henrik U, Georgia K Mitrousia, Pierre JGM de Wit, and Bruce DL Fitt. 2014. "Effector-triggered defence against apoplastic fungal pathogens." *Trends in plant science* 19 (8):491-500.

Tian, Miaoying, Edgar Huitema, Luis da Cunha, Trudy Torto-Alalibo, and Sophien Kamoun. 2004. "A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B." *Journal of Biological Chemistry* 279 (25):26370-26377.

Tian, Miaoying, Joe Win, Jing Song, Renier van der Hoorn, Esther van der Knaap, and Sophien Kamoun. 2007. "A Phytophthora infestans cystatin-like protein targets a novel tomato papain-like apoplastic protease." *Plant physiology* 143 (1):364-377.

Valli, Adrian, Gabriela Dujovny, and Juan Antonio García. 2008. "Protease activity, self interaction, and small interfering RNA binding of the silencing suppressor p1b from cucumber vein yellowing ipomovirus." *Journal of virology* 82 (2):974-986.

Vleeshouwers, VGAA, J Du, E Domazakis, A Chaparro-Garcia, X Lin, S Dong, D Wouters, MHAJ Joosten, S Kamoun, and RGF Visser. 2014. "Effector-driven breeding for broad-spectrum disease." Book of Abstracts XVI International Congress on Molecular Plant-Microbe Interactions.

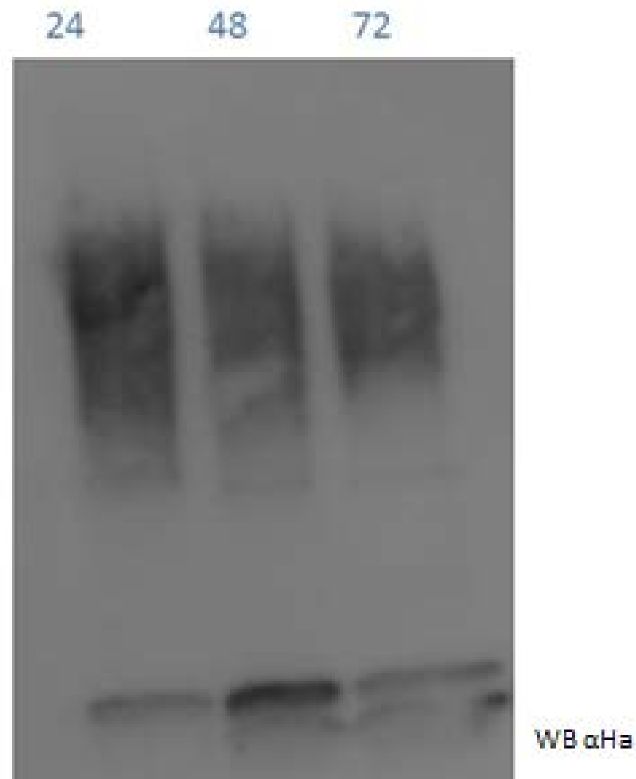
Wang, Guodong, Ursula Ellendorff, Ben Kemp, John W Mansfield, Alec Forsyth, Kathy Mitchell, Kubilay Bastas, Chun-Ming Liu, Alison Woods-Tör, and Cyril Zipfel. 2008. "A genome-wide functional investigation into the roles of receptor-like proteins in Arabidopsis." *Plant Physiology* 147 (2):503-517.

Win, J, A Chaparro-Garcia, K Belhaj, DGO Saunders, K Yoshida, S Dong, S Schornack, C Zipfel, S Robatzek, and SA Hogenhout. 2012. "Effector biology of plant-associated organisms: concepts and perspectives." Cold Spring Harbor symposia on quantitative biology.

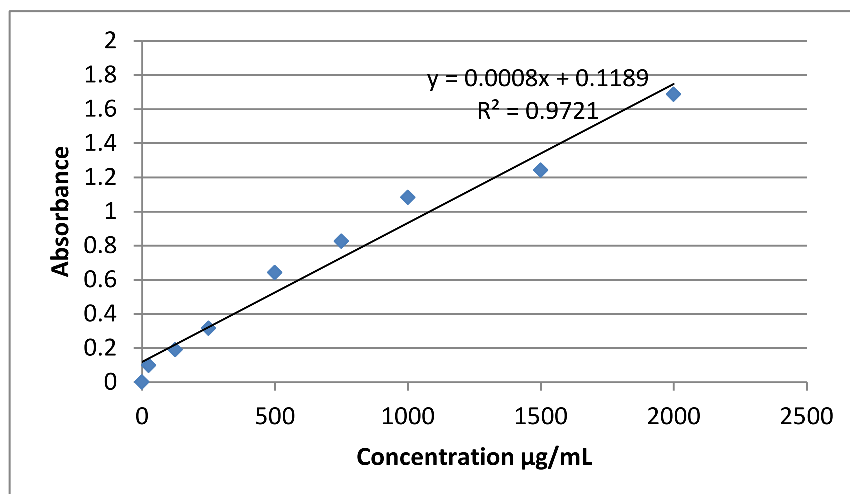
Zhu, Suxian, Ying Li, Jack H Vossen, Richard GF Visser, and Evert Jacobsen. 2012. "Functional stacking of three resistance genes against Phytophthora infestans in potato." *Transgenic research* 21 (1):89-99.

Appendices

Appendix A: Levels of HisHA protein expression and quantitative estimation

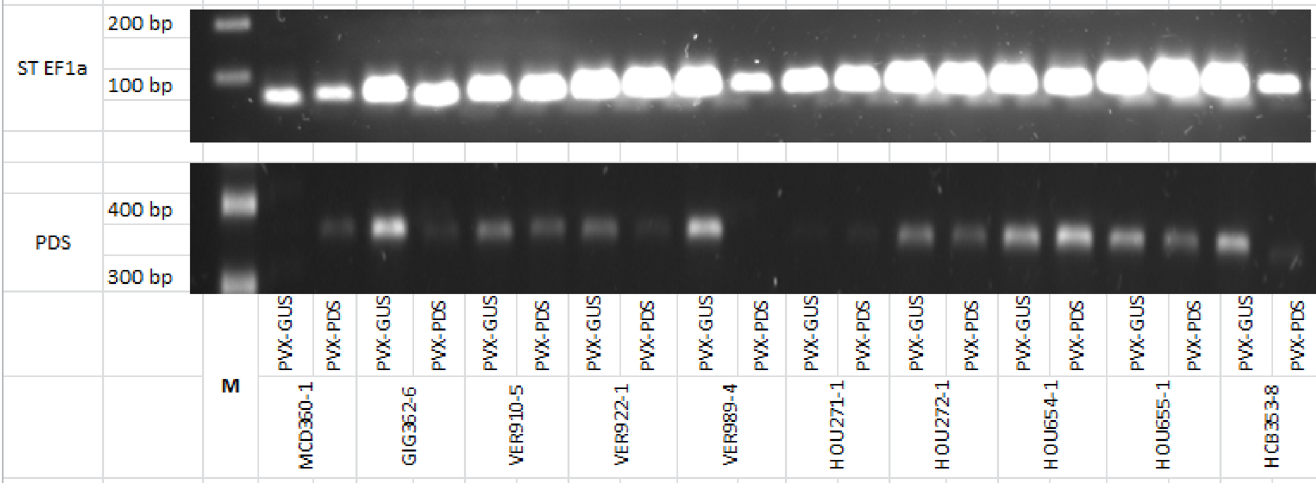


Supplementary figure 1: HisHA-Scr74-B3b protein expression at 24, 48 and 72-hour intervals. The 72-hour expression time point was selected for protein production.

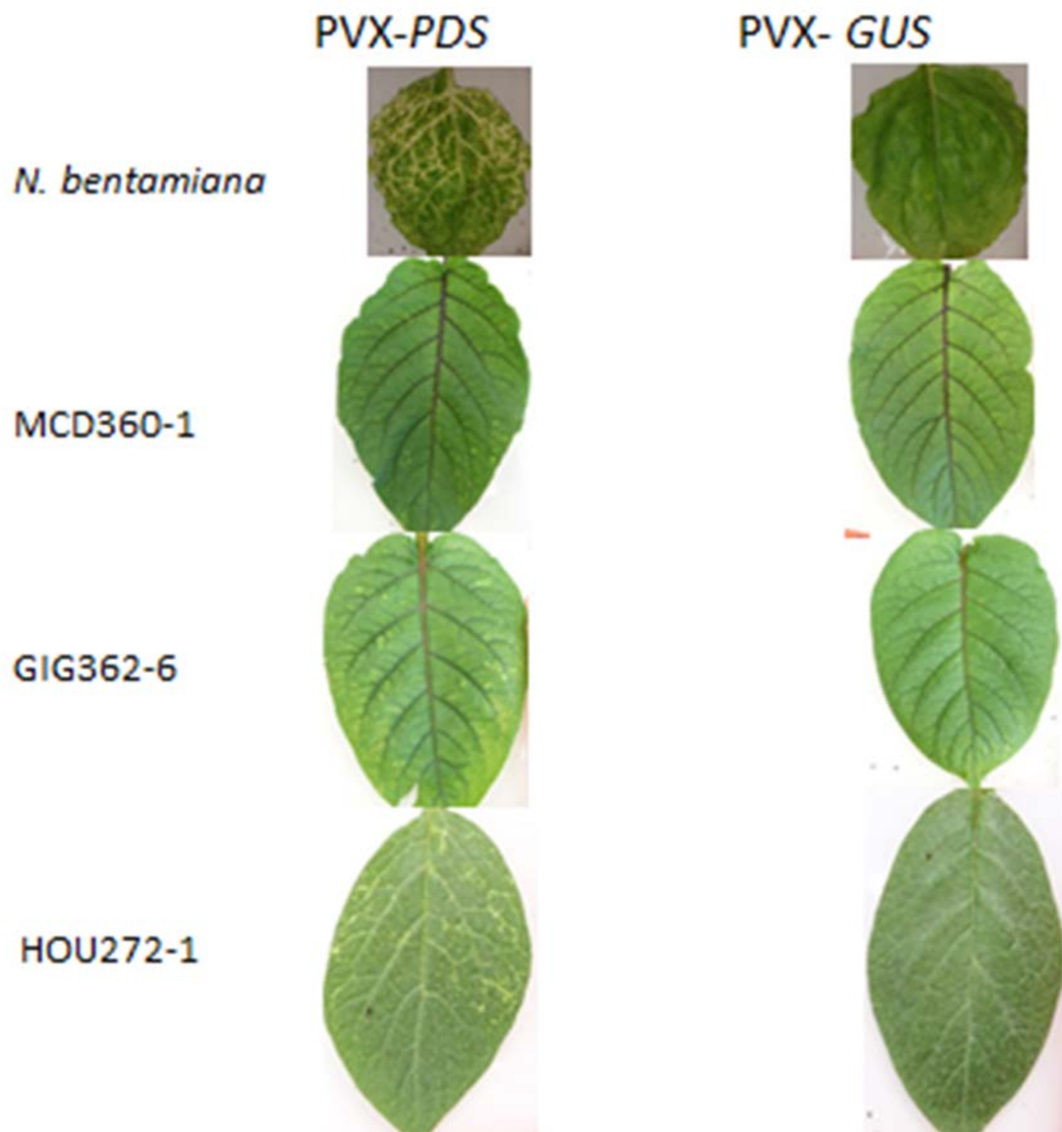


Supplementary figure 2: Amount of proteins produced in relation to absolute standards based on BCA quantification.

Appendix B: Estimation of *PDS* gene expression level

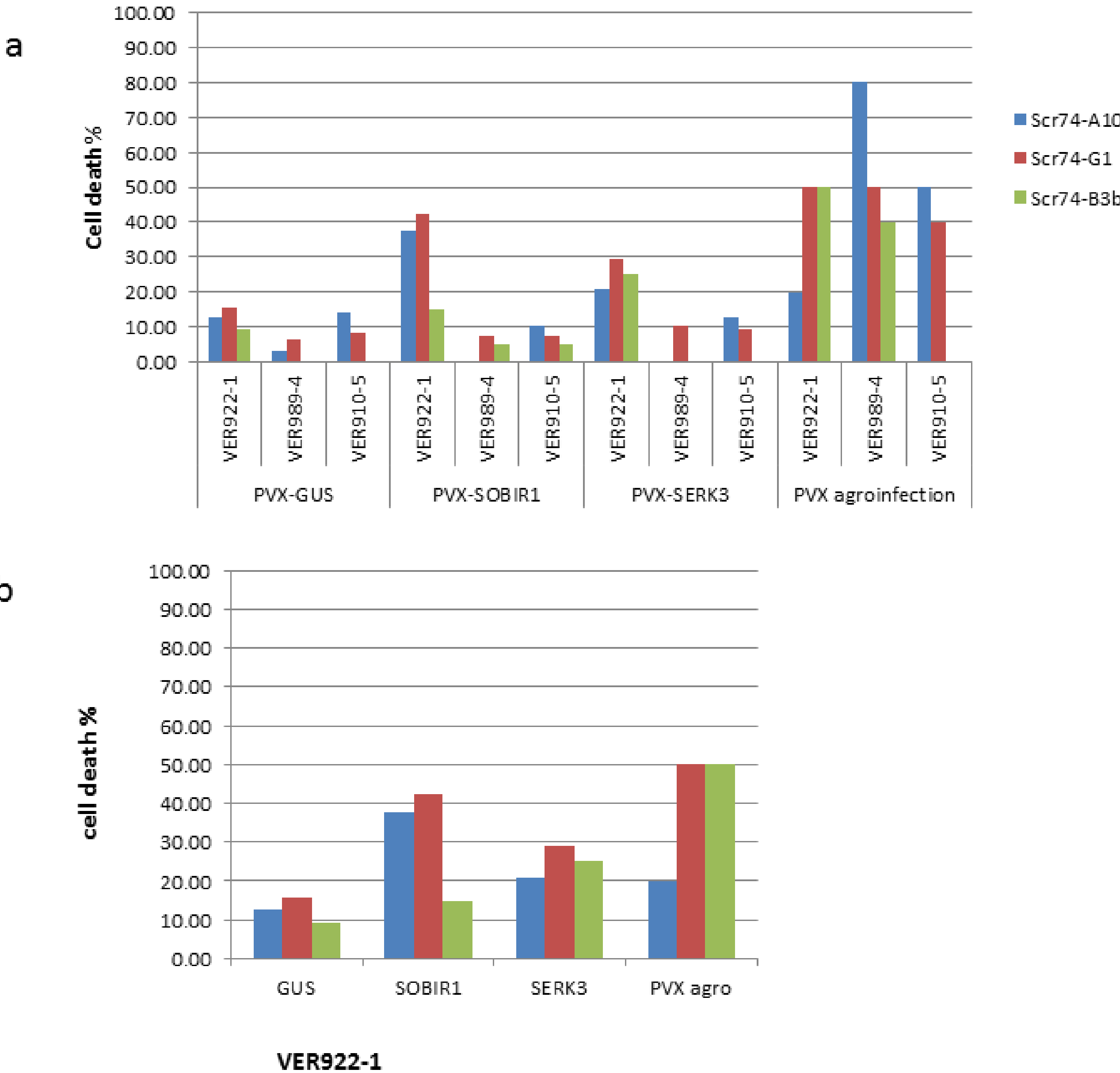


Supplementary figure 3: Expression level of *PDS* gene in relation to StEF1α (potato housekeeping gene) in PVX-*PDS* and PVX-*GUS* treated plants based on semi-quantitative q-PCR



Supplementary figure 4: Sample plants of visible photo-bleaching symptoms in PVX-*PDS* treated plants compared to PVX-*GUS* treated counterparts suggesting the silencing of *PDS* gene. Pictures were taken at 18 days post VIGS.

Appendix C: Cell death responses of protein infiltration and PVX agroinfection experiments



Supplementary figure 5: a) Cell death responses among *Solanum verrucosum* genotypes in protein infiltration (main experiment rep 1) and PVX agroinfection (previous experiment data). b) Protein infiltration and PVX agroinfection cell death responses in most responsive genotypes (VER922-1).